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Dependence on Na^+ of control of cytoplasmic pH in a facultative alkalophile

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Regulation of the cytoplasmic pH of *Exiguobacterium aurantiacum* is dependent on the presence of Na^+ in the medium. The data suggest that above 500 μM external Na^+ the cells are able to regulate the cytoplasmic pH by the operation of a Na^+ cycle involving a Na^+/H^+ antiport and a route for rapid Na^+ entry. Our data indicate that the rate of entry of Na^+ is subject to control by the cytoplasmic pH via feedback inhibition.

Exiguobacterium aurantiacum *Cytoplasmic pH* *Sodium cycle* *Na^+/H^+ antiport* *pH homeostasis* *pH jump*

1. INTRODUCTION

It has been proposed that the Na^+/H^+ antiport is essential for cytoplasmic pH (pH_i) homeostasis at alkaline external pH values in *Escherichia coli* [1] and in alkalophilic bacteria such as *Bacillus alcalophilus* [2], with the antiporter causing acidification of the cytoplasm by exchange of internal Na ions for external protons. Support for this mechanism of pH_i homeostasis has come from the study of a mutant of *Bacillus alcalophilus* which cannot grow when the medium pH is above 9.0 and which lacks the Na^+/H^+ antiport [3] as does a mutant of *Escherichia coli* which shows impaired growth at alkaline pH [4]. Unfortunately both mutant organisms show pleiotropic impairment of Na^+-linked transport processes [5,6] which weakens the evidence for the essentiality of the Na^+/H^+ transporter in pH_i homeostasis. Also a route of rapid Na^+ entry is required for continued operation of the Na^+/H^+ antiport, the two processes constituting a Na^+-cycle across the cell membrane [7] and this implies a requirement for Na^+ in the medium. Such a Na^+ requirement has not been observed in either *B. alcalophilus* or *E. coli* possibly owing to the universal presence of traces of Na^+ and a high Na^+ affinity in the carriers but has been demonstrated in an alkalophilic strain of *Bacillus firmus* [8,9]. It has also been pointed out [7] that if pH_i homeostasis is to be independent of the external Na^+ concentration then both the entry pathway for Na^+ and the Na^+/H^+ antiporter must be controlled, although no experimental evidence exists for such control of Na^+ entry.

This paper describes investigation of the role of Na^+ in pH_i homeostasis in *Exiguobacterium aurantiacum*, a facultative alkalophile [10] which requires Na^+ for growth at alkaline pH (unpublished). To investigate pH homeostasis and proton fluxes across the bacterial cell membrane the steady-state situation was perturbed by a rapid change in the external pH, and changes in pH_i were measured. This method is similar to the pulse-titration [11] or pH jump which has been used in investigations of growth [4] and other pH-dependent activities [12] in *E. coli*. As noted in [12] the change in ΔpH produced by the sudden change

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in external pH will cause an instantaneous change in the rate of proton extrusion processes and an opposite change in the rate of proton entry routes. In consequence pH_i will alter and the various proton transport processes will be regulated to establish a new steady-state ΔpH and pH_i. The data presented here demonstrate the need for Na^+ in the response of *E. aurantiacum* to a pH-jump and that in the presence of a sufficient external Na^+ concentration the organism exhibits rapid and effective pH_i homeostasis. These data and those for a Na^+-concentration jump are consistent with the hypothesis of a Na^+-cycle being responsible for pH_i homeostasis and indicate control of the cycle by pH_i.

2. MATERIALS AND METHODS

2.1. *Organism*

Exiguobacterium aurantiacum gen.nov., sp. nov. (NCIB 11798) [13], previously referred to as BL77/1, was isolated at 20°C from potato-processing effluent [10]. The strain was maintained both as freeze-dried cell suspensions and on slopes of PPYG medium as in [10].

2.2. *Growth conditions*

The organism was grown overnight at 37°C on PPYG medium [10]. The overnight culture was harvested by centrifugation (12000×*g*, 8°C, 5 min) and washed twice in 50 mM 2-*N*-cyclohexylaminoethanesulphonic acid (CHES)–KOH (pH 9.0). The washed cells were diluted to an A_{680} of about 0.3 (an A_{680} of 2.25 is equivalent to 1 mg bacterial dry wt/ml) with SD-medium and reincubated at 37°C in an orbital shaker (Gallenkamp) at 250 rev./min for 2 h. The SD-medium contained, per l, K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; glucose, 5 g; yeast extract, 1 g; $FeSO_4$, 40 mg; $CaCl_2 \cdot 6H_2O$, 60 mg; $MnSO_4 \cdot 4H_2O$, 6 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.8 mg; $CuSO_4 \cdot 5H_2O$, 8 mg; $CoCl_2$, 0.8 mg; $ZnSO_4 \cdot 7H_2O$, 8 mg; and 50 mM CHES–KOH buffer (pH 9.0). The culture pH was maintained at pH 9.0 by addition of aliquots of 2 N KOH at 30-min intervals. Exponential phase cells were harvested and washed 3 times in 50 mM CHES–KOH buffer (pH 9.0) containing 1 mM $MgSO_4$ (CHES was purified by re-crystallization of a saturated solution to reduce the content of contaminating Na^+). The final cell suspension was kept on ice until required.

2.3. *Measurement of pH_i and $\Delta\psi$*

ΔpH and $\Delta\psi$ were determined using centrifugation-based assays as in [14]. The transmembrane distributions of the weak base [^{14}C]methylamine and the lipophilic cation [^{14}C]tetraphenylphosphonium (TPP^+) were used to measure ΔpH and $\Delta\psi$, respectively. Cells at 2 mg dry wt/ml were incubated at 25°C in 50 mM CHES–KOH buffer (pH 9.0) in the presence of 10 mM glucose, 1 mM $(NH_4)_2SO_4$, [^{3}H]inulin (100 μM; 3.97 Ci/mmol), and either [^{14}C]TPP^+ (16 μM; 9.4 Ci/mol) or [^{14}C]methylamine (22 μM; 4.6 Ci/mol). The intracellular water volume, as determined in [15] was 2.09 μl/mg dry wt cells. The concentration of contaminating Na^+ in the incubations was measured using a flame photometer (EEL Evans Electroselenium).

2.4. *Materials*

All chemicals were purchased from BDH (AnalaR), except inulin and CHES which were obtained from Sigma, and yeast extract which was obtained from Pifco laboratories. All radiochemicals were purchased from Amersham International. KOH was of aristar grade.

3. RESULTS

3.1. *Effect of Na^+ on pH_i during shifts in pH_e*

Cell suspensions of *E. aurantiacum* maintained an intracellular pH of 8.3 in the absence of added Na^+ at pH_e 8.85. Addition of KOH to increase pH_e by 0.55 units to pH_e 9.4 provoked an increase of pH_i of similar magnitude (fig.1). The increase in pH_i was initially rapid and then slowed until a new steady state was reached 3 min after the change in pH_e. In the presence of 1 mM Na^+ the change in pH_i occasioned by the increase in pH_e was smaller and the new steady state was achieved almost instantly (fig.1). Thus the presence of Na^+ appeared to allow control of pH_i to a lower value and to accelerate the approach to the new steady state. When cells were exposed to Na^+ addition and a pH jump simultaneously the effect was to reset pH_i at a lower value identical to that obtained when Na^+ was present throughout (fig.1). This suggests that Na^+ entry is very rapid since the regulation of pH_i

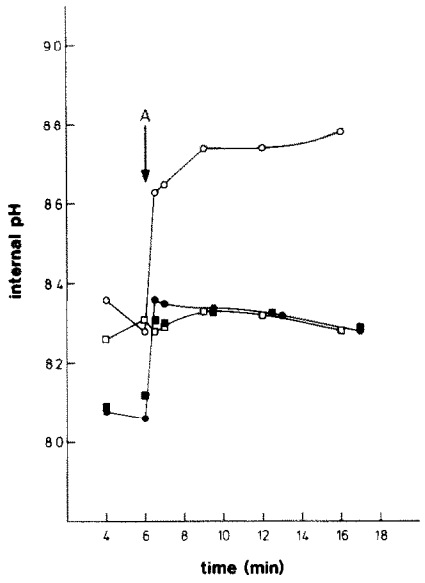

Fig. 1. Effect of Na^+ on pH_i during pH_e shift. Cells were incubated at 2 mg dry wt/ml in 50 mM CHES-KOH buffer (pH 8.85) containing 1 mM $(NH_4)_2SO_4$ and 10 mM glucose, in the presence (●,■) and absence (○,□) of 1 mM NaCl. Samples were taken at intervals and pH_i assayed by the transmembrane distribution of [^{14}C]methylamine (22 μM; 4.6 Ci/mol). (A) The addition of an aliquot (50 μl) of 2 N KOH (○,●) or 2 N NaOH (□,■) which raised pH_e to 9.4 and 9.48, respectively. The intracellular pH was again measured at intervals. The external pH was monitored throughout.

requires the lowering of pH_i by exchange of internal Na^+ for external H^+.

3.2. *Effect of jumps in Na^+ concentration on pH_i*

Addition of Na^+ to cells incubated in low-Na^+ medium at high external pH provoked a rapid acidification of the cytoplasm (fig.2). Both the rate and the initial extent of the fall in pH_i were dependent on the new external Na^+ concentration. When addition raised the external Na^+ to less than 500 μM the final steady state pH_i was markedly dependent on Na^+ concentration; above this concentration the steady state pH_i was unaffected by the concentration of Na^+. Above 500 μM Na^+, however, there was a transient over acidification of the cytoplasm, the extent of which was dependent on the Na^+ concentration (fig.2). Thus above 500 μM Na^+ cells exhibit regulation of pH_i which is dependent on Na^+flux, but independent of Na^+ concentration.

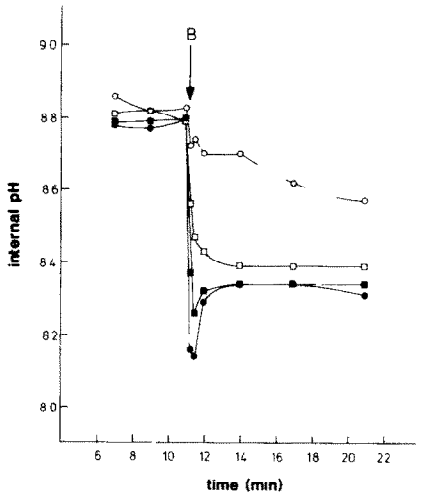

Fig. 2. Effect of Na^+ concentration shifts on pH_i. Cells were incubated in the absence of added Na^+ at pH 9.0, and KOH was added after 7 min incubation to raise pH_e to 9.65 as in fig.1. (B) Addition of Na^+ (as NaCl): 100 μM Na^+ (○); 500 μM Na^+ (□); 2 mM Na^+ (■); 10 mM Na^+ (●). pH_i was measured as described for fig.1.

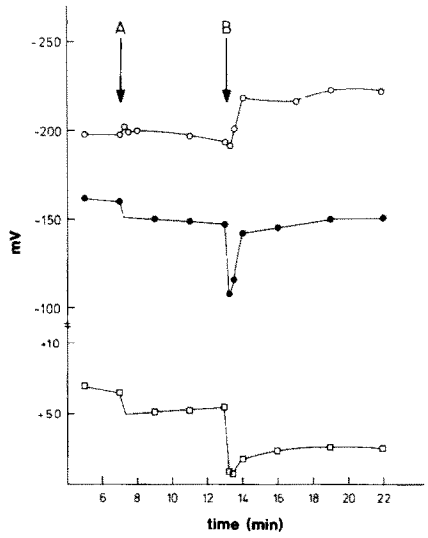

Fig. 3. Effect of Na^+ on $\Delta\psi$. This was a parallel incubation to that described in fig.2 except that the $\Delta\psi$ was monitored from the transmembrane distribution of [^{14}C]TPP^+ (16 μM; 9.4 Ci/mol) as described in section 2. (A) Addition of 2 N KOH, raising pH_e from 9.0 to 9.6. (B) Addition of 10 mM Na^+ (as NaCl) (○). The results from fig.2 of the effect of 10 mM Na^+ on pH_i are shown in mV (□). The total protonmotive force, Δp ($\Delta\psi$ − 59 pH) was calculated from the sum of the above measurements (●).

3.3. *Effect of Na^+ and pH_e on the membrane potential*

The membrane potential ($\Delta\psi$) of cells incubated in the absence of added Na^+ was not affected by an alkaline pH shift with KOH (fig.3). Subsequent addition of Na^+ (10 mM) and the consequent reduction of pH_i resulted in an increase in $\Delta\psi$ of 25 mV. This increase in $\Delta\psi$ followed the restoration of pH_i to a more acidic value and compensates for the decrease in the ΔpH component of the protonmotive force. Overall the protonmotive force remained constant (fig.3). A caveat must be placed against these data, TPP^+ was used as a probe of $\Delta\psi$ and may be unreliable in some circumstances [16]. Despite this reservation it does appear that $\Delta\psi$ increases after the Na^+-dependent restoration of pH_i to a more acidic value.

4. DISCUSSION

The results presented here show that external Na^+ is essential for acidification in pH_i homeostasis in *E. aurantiacum* (fig.1,2), these being the first observations which directly demonstrate the effects of Na^+ on pH_i regulation in an alkalophilic bacterium. Furthermore, the time scale of the responses shows that the entry pathway for Na^+ must allow rapid entry of Na^+ at a rate comparable to, or greater than the rate of the Na^+/H^+ antiport.

Below 500 μM external Na^+ it appears that the rate of Na^+ entry is the limiting factor in both the speed of response to a changed pH_e and in the maintenance of a steady-state pH_i lower than pH_e but above 500 μM external Na^+ the bacterium is able to respond rapidly and maintain effective pH_i homeostasis.

The Na^+-jump experiments provide further information about the entry pathway and its control. Authors in [7], in discussing the need for control of both halves of the Na^+ cycle in order to provide pH_i homeostasis suggested control of the Na^+ entry pathway. The overshoot of pH_i shown in the Na^+ jump experiments (fig.2) rules out internal Na^+ or external pH as the controlling parameter as either would provide instantaneous control of the Na^+ entry pathway. It would appear that it is the cytoplasmic pH (pH_i) which controls the Na^+ entry pathway since, on account of the buffer capacity of the cytoplasm and the need for protons to enter via the Na^+/H^+ antiport, adjustment of pH_i will lag behind Na^+ entry and during this lag, excess Na ions will enter before the Na^+ entry pathway is shut-off by the lowered pH_i. The excess internal Na^+ causes the overshoot in lowering pH_i as it leaves via the Na^+/H^+ antiport. Subsequently, the stress of high external pH, as well as respiration and ATPase driven proton jumps will operate to raise pH_i as the oscillation settles to the steady-state pH_i with the Na^+/H^+ antiport also regulated to a low level of activity. Control of both sectors of the Na^+ cycle not only limits Na^+ entry and hence maintains the internal Na^+ concentration at a low level, but will minimize the steady state rate of Na^+ cycling thus also minimizing the energy requirement for pH_i homeostasis.

However, the mechanism thus proposed leaves two problems unanswered, firstly that of overall electrical charge balance and secondly, the source of energy for driving the cycle. Authors in [7] suggested charge balance by anion entry but the alternative of cation efflux is also worth considering. Of the internal cations, K^+ is the most likely candidate, and if the K^+ efflux were linked to Na^+ entry on an antiporter this could provide part of the energy for driving the Na^+ cycle, alternatively either of the sodium transport steps could be energised by ATP hydrolysis.

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